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Molecular Cloning of Haemagglutinin Gene Isolated from Egyptian H5N1 Strain

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Abstract: The spread of highly pathogenic avian H5N1 influenza viruses since 1997 and their virulence for poultry and humans has raised concerns about their potential to cause an influenza pandemic. Early detection of newly circulating viral strains as well as vaccination offers the most viable means to combat a pandemic threat. However, it will be a challenge to produce, distribute and implement a new vaccine if a pandemic spreads rapidly. The current study aimed to isolate the full length hemagglutinin (H5) gene *via* Polymerase Chain Reaction (PCR), subsequently clone the isolated gene and, finally, sequence the amplified fragment in order to identify the restriction recognition sequences that should be considered for further expressional cloning experiments. In order to achieve these objectives, a real- time PCR protocol specific to the Egyptian H5N1 isolates has been developed and tested on both *in silico* and *in vitro* levels. Also, full length H5 gene has been isolated *via* PCR. Moreover, isolated H5 gene has been cloned into TOPO10 molecular cloning vector and partially sequenced. Finally, bioinformatics analysis of generated DNA sequence data has been carried out in order to construct the phylogenetic tree of the studied local H5N1 isolate as well as to determine the restriction recognition sequences inside the sequenced length of H5 gene in order to be avoided in further expression cloning experiments.

Key words: Molecular cloning · Haemagglutinin Gene · Egypt-H5N1 Strain · Real · Time PCR

INTRODUCTION

Avian influenza (AI) is a contagious viral disease affecting poultry and also humans. In recent years there has been a reemergence of highly pathogenic avian influenza (HPAI) viruses causing severe outbreaks in various parts of the world [1] including Middle East and Eastern European countries [2, 3]. To date, H5N1 virus caused huge economic losses for poultry industry and overall national economy of some countries. Moreover, since 1999, the concern on potential human influenza pandemic increased significantly as sporadic and serious outbreaks occurred after transmission of avian species to humans [1,4], leading to severe and fatal infections. The first human case of A (H5N1) avian influenza in Egypt was diagnosed in 2006 [5]. Raising poultry in households is common in Egypt, where community awareness of the risks of avian influenza seems to remain low. In December 2014, the Egyptian veterinary authorities indicated that the capacity of veterinary services to investigate outbreaks and carry out surveillance was challenged, mainly by limited resources (e.g. manpower, vehicles and equipment). The authorities faced difficulties in controlling the disease due to the high density of commercial and household poultry flocks, the intensity of bird movements and low awareness of the disease in the rural population. In Egypt, the total number of infected cases was 344 out of them 114 deaths were recorded, the mortality rate of H5N1 infected cases up to 2015 was 33%. [5]. H5N1 has the potential of becoming a major human threat due to its inherent antigenic variation, high mortality and morbidity [6].

Therefore, it is important to control and prevent the infection and spread of H5N1 AIVs to protect the health of both animals and humans. Thus, a comprehensive strategy for effective control and prevention of H5N1, including diagnostics, surveillance and vaccination must be developed.

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Diagnostic approaches for H5N1 virus include virus isolation, immunochromatographic and reverse transcriptase (RT) PCR [7].

DNA vaccines offer a number of advantages over conventional vaccines [8]. However, Owing to error-prone viral RNA polymerase activity, influenza virus H5 gene is subject to a very high rate of mutation [9]. Therefore, development of molecular vaccine based on H5 gene sequences requires the optimization and establishment of methods and techniques for the fast isolation of the target antigenic H5 gene. Hence, the current study aimed at characterization, molecular cloning and sequencing of the full length hemagglutinin gene isolated from local H5N1 field isolate.

MATERIALS AND METHODS

Sample Preparation: Two Egyptian H5N1 viral isolates named A/chicken/Qalyobia/Egypt/H5N1/2012/1andA/ chicken/Sharkia/Egypt/H5N1/ 2012 have been isolated and propagated by the use of specific pathogen free (SPF) standard approach for testing the diagnostic protocol. The local H5N1 isolate A/chicken/ Qalyobia/Egypt/ H5N1/2012/2 has been used for the isolation of the full length H5 antigenic gene.

Viral RNA Extraction: Nucleic acid of the propagated viral isolates has been carried out *via* QIAamp Viral RNA Mini Kit (Cat#52904, Qiagen, Germany) as described by the manufacturer protocol. Viral isolates were also tested by the haemaglutinine neutralization test in order to ensure that the propagated viruses belong to H5N1 virus.

Reverse Transcriptase PCR Analysis: Six microliters of extracted RNA (40μg/μl) were added to 6 μl of avian myeloblastosis virus (AMV) reverse transcriptase (Promega, USA), 15 μM of Uni12 and random avian influenza virus oligonucleotide primers [10]. Reaction mixture was incubated at 25°C for 5 min, 42°C for 10 min, 50°C for 20 min and 85°C for 5 min and was then hold at 4°C until amplification.

***In Silico* Analysis:** Egyptian H5 c-DNA sequences have been downloaded from the flu database (www.ncbi.nlm.nih.gov) and a sequence database has been constructed by the use of Bioedit software (www.bioedit.com). Sequence alignment of the collected c-DNA sequences has been carried out using the same program. Conserved regions were used as a template for H5 primer design by the use of primer express software (Applied Biosystem, USA). Designed oligonucleotide primers have been tested *in silico* against the downloaded viral c-DNA sequences in order to examine the accuracy of designed primers.

Bioinformatics Analysis of Generated H5 Gene Sequence: Generated c-DNA sequences have been aligned and used for building the phylogenetic tree of the studied H5 isolate. Finally, the bioedit software was used for the identification of restriction recognition sequences of the generated partial H5 gene c-DNA sequences.

Optimization of Diagnostic Real Time PCR Protocol: Several real time PCR trials have been carried out in order to optimize the diagnostic protocol that employs the designed primers and to test the efficiency of the designed diagnostic protocol *in vitro*. Master mix components were 2x universal standard SYBR Green chemistry (Applied Biosystem, USA), 150 pmoles of forward and reverse oligonucleotide primers (Applied Biosystem, USA). Thermal cycler program was 95°C for preheating step for 30 seconds, 35 cycles of 15 seconds at 95°C and 60°C for 1 minute for both annealing and extension steps. Melting curve analysis has been carried out at 65-90°C on 2°C intervals.

Sensitivity Test: In order to test the sensitivity of the proposed real time PCR protocol, serial dilutions of the initial viral nucleic acid concentration (40μg/μl) was prepared. These serial dilutions were used as a template against the designed oligonucleotide primers in a real time PCR format as described above.

Isolation of Full Length H5 Fragment: Isolation of the Full length of H5 gene has been carried out *via* PCR as described by [11] with some modifications. Using KAPA BIOSYSTEM Taq (KAPA2G Fast PCR Kit, Jena Biosciences, Germany) as follow; 5μl of 5X KAPA2G, 0.5μl of MgCl2 (25 mM), 0.5 μl of dNTPs Mix., 1.25 mM of both forward and reverse primer, 1μl of 10ng Template c-DNA and finally 0.1U of KAPA2G Fast DNA polymerase. Thermal cycler program (BIOMETRA, Germany) includes initial denaturation at 95°C for 3 min., 40 cycles of 95°C for 15 sec., 50°C for 30 sec. and 72°C for 5 min., final extension step was performed at 72°C for 15min.

Agarose Gel Electrophoresis: Visualization and documentation of generated PCR full length gene fragment has been carried out by the use of agarose gel electrophoresis (1.2%). Fractionated PCR bands have been visualized by the use of UV trans-illuminator (UVP, UK).

Elution of Full Length Gene: Target PCR product (~ 1800 bp) has been eluted from the agarose gel by the use of gel elution kit (QIAGEN, Germany) and fractionated again at the same conditions described above in order to ensure the obtaining of target length prior to sequencing analysis.

Sequencing of the Isolated Full Length H5 Gene: Amplified full length H5 PCR product has been sequenced at sequencing facilities of Macrogen, South Korea.

Cloning of the Full Length H5 Gene: Full length H5 gene has been cloned into pCR2.1 cloning vector using TOPO10 cloning kit (Applied Biosystem, USA) as described by the manufacturer protocol.

RESULTS AND DISCUSSION

***In Silico* Analysis:** Two types of oligonucleotide primers have been used at the present study. First type are previously published N1 gene oligonucleotide primers used to ensure that the studied isolates are belongs to type A N1 subtype of avian influenza viruses [11]. Second type represents oligonucleotide primers designed at this study (Table 1) to confirm that they are belongs to H5N1 AIV subtype. In order to ensure the specificity and accuracy of the designed oligonucleotide primers, sequence alignment of designed oligonucleotide primers have been performed against Egyptian H5N1 nucleotide DNA sequences. Generated data confirmed a high specificity and accuracy of the tested primers against the Egyptian H5N1isolates in compared to previously published oligonucleotide primers designed by [11]. As shown in figures 1 and 2 no mismatches have been detected between oligonucleotide primers designed at this study and DNA sequences of the tested Egyptian H5N1 isolates. These data confirmed that designed oligonucleotide primers specific to local H5N1 isolates and hence, appropriate for improves the efficiency of proposed diagnostic protocol.

Optimization of Real Time PCR Protocol: In order to test the efficiency and accuracy of the designed oligonucleotide primers, a specific real time PCR protocol has been optimized. Generated Cycle threshold (C.t.) values showed that oligonucleotide primers designed at this study have earlier C.t. values compared to the previously published protocols [11]. Cycle threshold (C.t.) values produced at this experiment (Table 2 and Figure 3) indicated the efficiency of the optimized diagnostic real time PCR protocol to detect the local Egyptian H5N1 viral isolates. Moreover, melting point analysis of the amplified product confirmed the specificity of proposed protocol without any non- specific product and/or primer- dimer structure (figure 4). This result is in accordance with the results generated by [12] who confirmed that the sensitivity of diagnostic real time PCR assays that are designed specifically for local H5N1 Egyptian isolates are higher than using generic RT-PCR protocols.

Table 1: Oligonucleotide primers used at this study

Primer name	Primer sequence	Reference
H5 MUST 1F	CGACAAGGTCCGACTACAGC	This study
H5 MUST 1 R	TGCGGGTAGTCATACGTTCC	This study
H5 MUST 2R	ACTGCGGGTAGTCATACGTTC	This study
MF3	TGATCTTCTTGAAAATTTGCAG	Pyungporn *et al.,* 2006
MR1+	CCGTAGMAGGCCCTCTTTTCA	Pyungporn *et al.,* 2006

Table 2: Cycle threshold values generated by oligonucleotide primers used at this study

Primer Combinations	H5MUST1F/ H5MUST1R	H5MUST1F/ H5MUST2R	M1F/M1R
Ct value	27	19.42	25.16

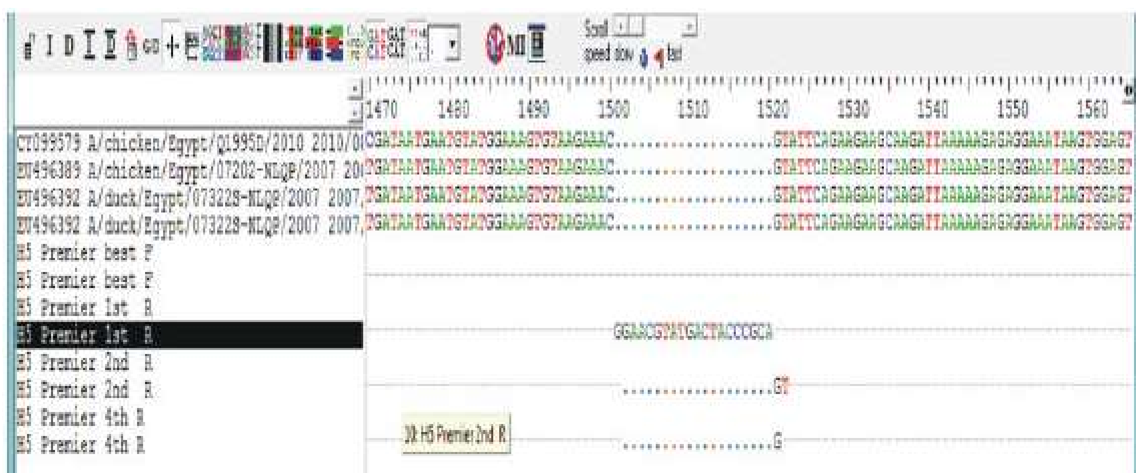

Fig. 1: Sequence alignment of oligonucleotide primer H5MUSTR1 against Egyptian H5 DNA sequences.

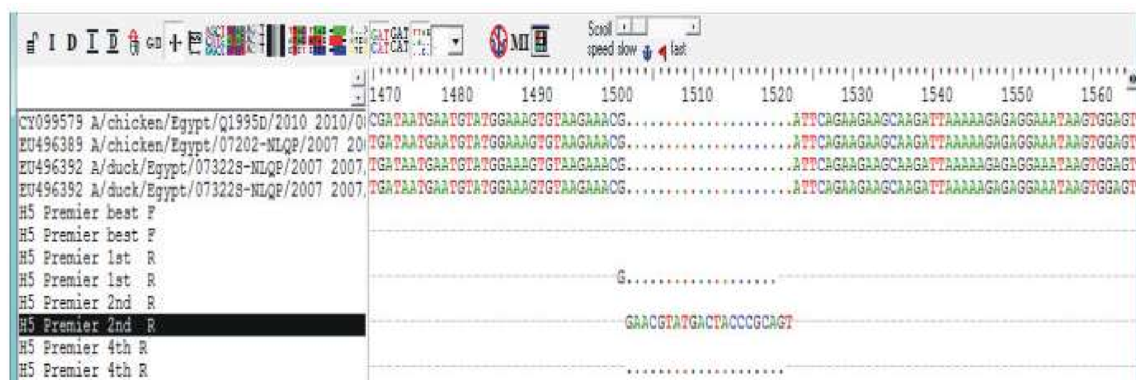

Fig. 2: Sequence alignment of oligonucleotide primer MUST2 R2 against Egyptian H5 DNA sequences.

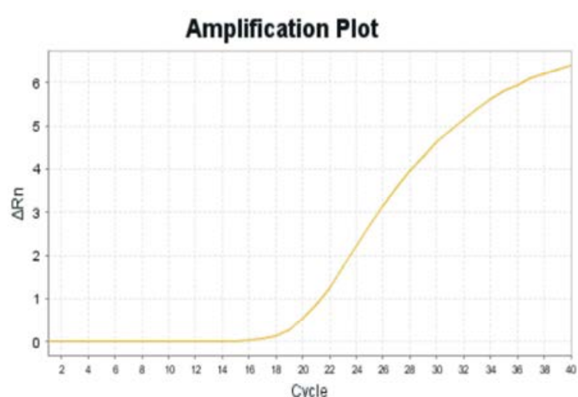

Fig. 3: Amplification plot of H5 MUST1 F and H5 MUST2 R1 primers against the target Egyptian H5N1 isolate.

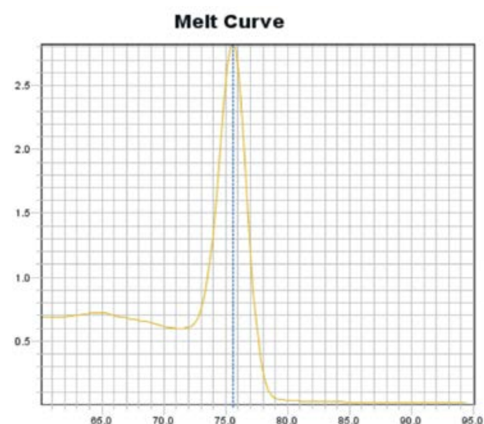

Fig. 4: Melting curve analysis of H5 MUST1 F and MUST2 R1 primers against the target Egyptian H5N1 isolate.

Sensitivity Test: In order to examine the efficiency of developed protocol, a sensitivity test has been carried out using serial dilutions of the original isolated RNA concentration (40ug/µl). Results of this experiment illustrated a gradual increase of the Ct values parallel to the increase of the dilution factor (Table 3 and Figure 5).

Table 3: List of Ct values produced by real time PCR sensitivity test analysis of sample 1 against all tested primers

Primer Dilution	H5MUST1F/ H5MUST1R	H5MUST1F/ H5MUST2R	M1F/M1R
1/100	20.53	20.6	24.8
1/1000	24.2	23.9	27.2
1/10000	28.9	27.7	32.00
1/100000	30.6	29.9	32.8
1/1000000	33.2	33.7	33.5

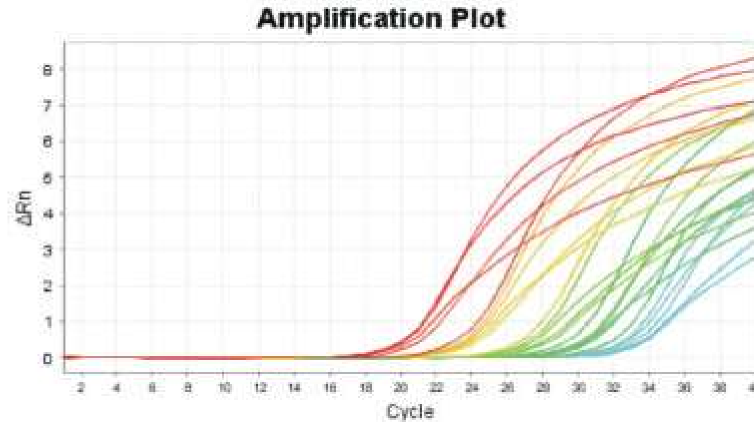

Fig. 5: Over all amplification plots of all primers against serial dilutions of standard viral RNA.

Gradual increase of Ct values in response to the dilution of RNA template indicates a strong dynamics and efficiency of the proposed diagnostic real time PCR protocol as stated in other studies [11]. Furthermore, it has been stated that the control and eradication of H5N1 spread by specific rapid diagnostic testing, vaccination and culling of poultry represents the cornerstone to overcome the risk of human infections and fatalities in Egypt. However, continuing viral evolution represents a daily challenge for diagnostic measures which are at the root of all efforts to control the situation. Finally, characterization of newly circulating isolates and updating the amplification-based diagnostic tools, such RT-PCR, is essential to improve the eradication and control strategies [12].

Isolation of Full Length H5 Fragment: The full length H5 antigenic gene of the studied Egyptian H5N1 viral isolate (A/chicken/ Qalyobia/Egypt/ H5N1/2012/2) has been isolated *via* PCR. The first step is to transfer c-DNA copy of each genomic fragment by the use of H5N1 random primer (UNI-12) mentioned at the materials and method section. The advantage of using this random H5N1 oligonucleotide primer attributed to the specificity of it to all flanking regions of each of the eight fragments of the type A avian influenza viruses. Results of this experiment showed that the expected size of the full length H5 gene

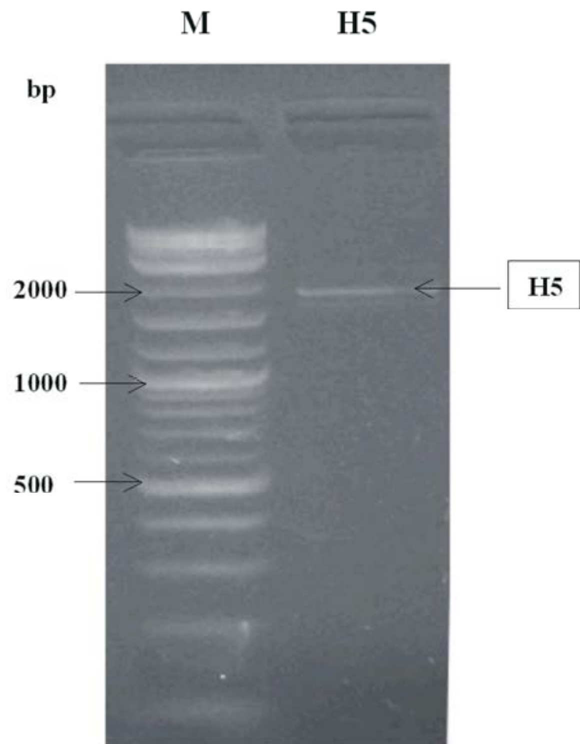

Fig. 6: Size fractionation of full length H5 gene on 1.2% Agarose gel electrophoresis before elution,
M.: Marker 100 - 3000bp
H5: PCR product of full length H5gene.

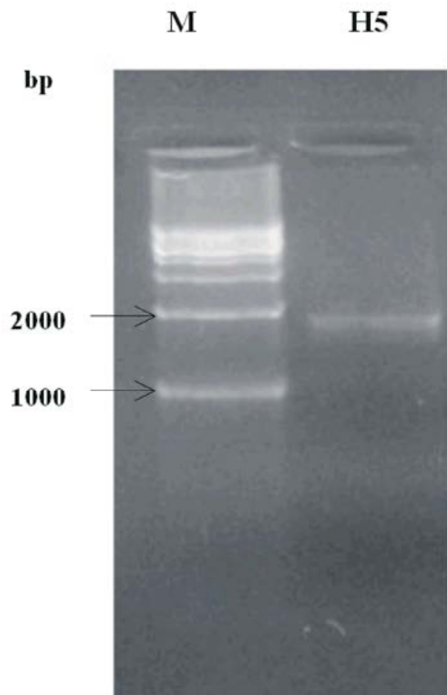

Fig. 7: Size fractionation of full length H5 gene on 1.2% Agarose gel electrophoresis after elution,
M: Marker 1k b
H5: PCR product of full length H5gene.

has been obtained (Figure 6 and 7). As described by Hoffmann *et al*. [10] the size of full length H5 gene using the recommended specific primers that are flanking the coding sequences is 1807 bp. The size of generated PCR product is in accordance with the H5 gene generated by Hoffmann *et al*. [10] who documented that the used oligonucleotide primer described at his research are appropriate for the amplification of full length HA genes belongs to all influenza A viral subtypes. This experiment is also very important for further sub-typing, sequencing and to construct expression plasmids for molecular vaccine purposes.

Sequencing Analysis of Isolated Full Length Genes: Eluted full length H5 gene (Figure 7) has been sequenced in order to determine the phylogenetic relationship of the studied isolate. DNA sequencing of the H5 gene represents the most powerful and reliable tool for studying the evolution of avian influenza viruses [13]. However, due to technical reasons DNA sequencing data generated partial length DNA sequences (Figure 8). Therefore, additional trials to sequence the entire full length antigenic gene that has been isolated should be performed.

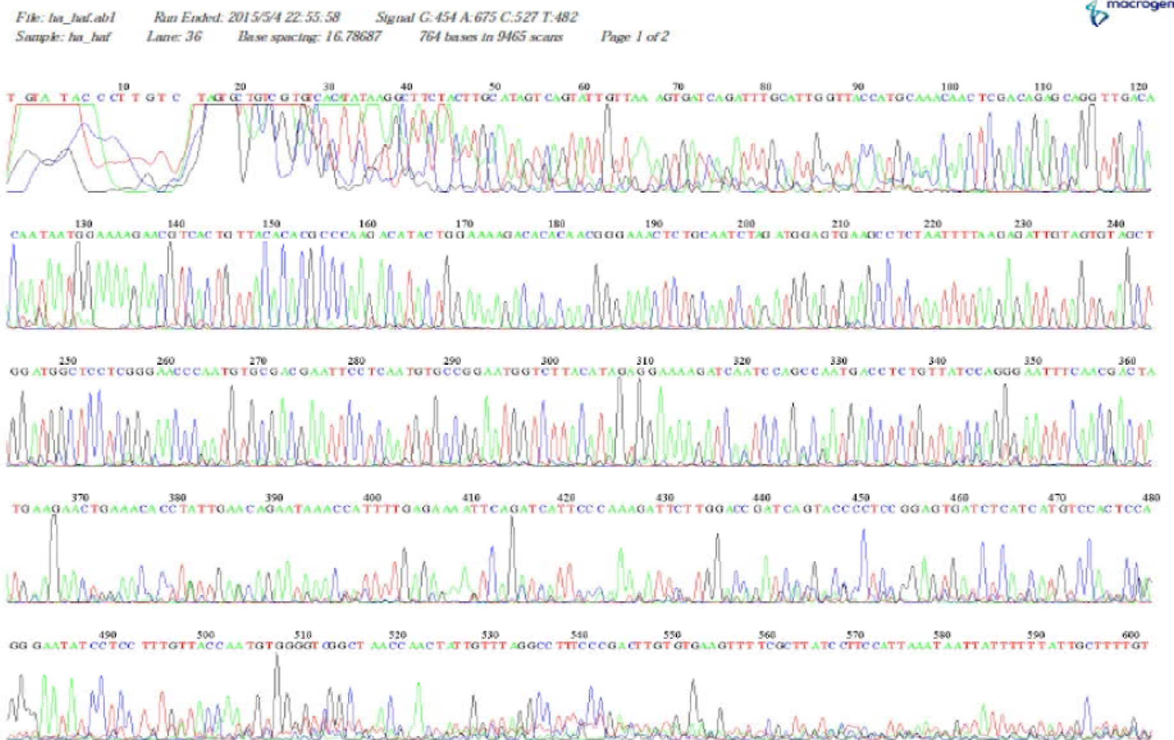

Fig. 8: Partial DNA sequencing data of H5 gene.

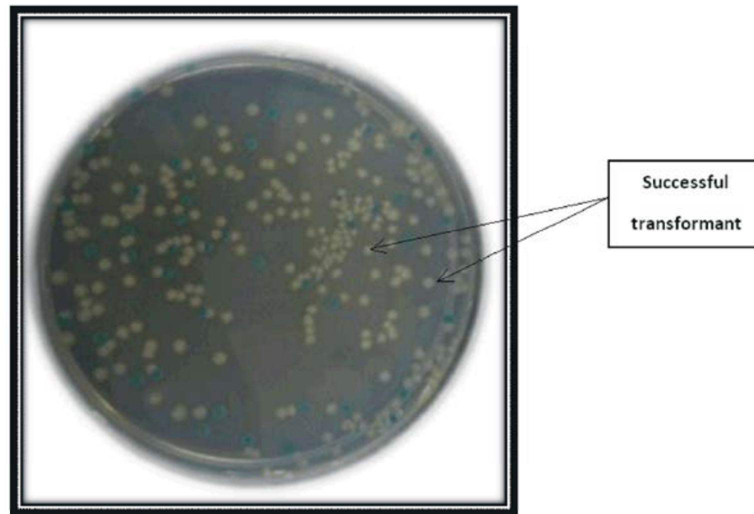

Fig. 9: Selection of successful transformants (White colonies).

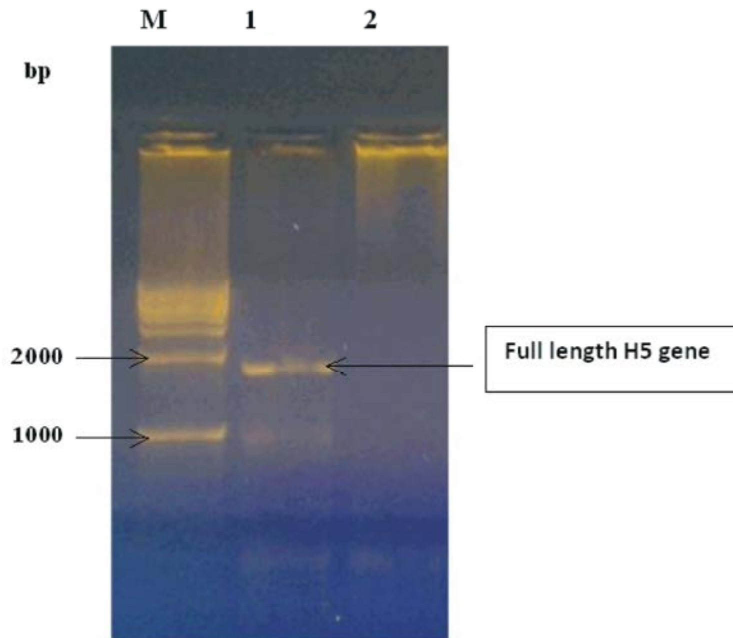

Fig. 10: Amplification of the target insert (full length H5 gene) using cloning vector present at successful clone as a template,
M: Marker 1kb ladder
lane 1: Amplified full length H5 gene generated from successful clones;
lane 2: amplification product (negative results) of negative control cells.

Bioinformatics Analysis of Generated H5 Gene Sequence: Bioinformatics analysis have been used for constructing a phylogenetic tree of studied Egyptian H5N1 isolate (A/chicken/Qalyobia/Egypt/ H5N1/2012/2) that has been used for the isolation of full length antigenic gene utilizing the data generated from DNA sequencing of corresponding H5 gene among other international H5N1 isolates. As shown in figure 11, phylogenetic data revealed that the studied isolate was closely related to the Egyptian isolate Egypt-MOH-NCR-7305. Finally, *in silico* analysis of sequencing data has been carried out in order for identify the restriction recognition sequences inside the studied H5 gene. Restriction enzymes that must be avoided during further cloning experiments have been partially identified (Figure 12).

Fig. 11: Phylogenetic analysis of the studied isolate (ha_haf) and other H5N1 strains recorded at NCBI.

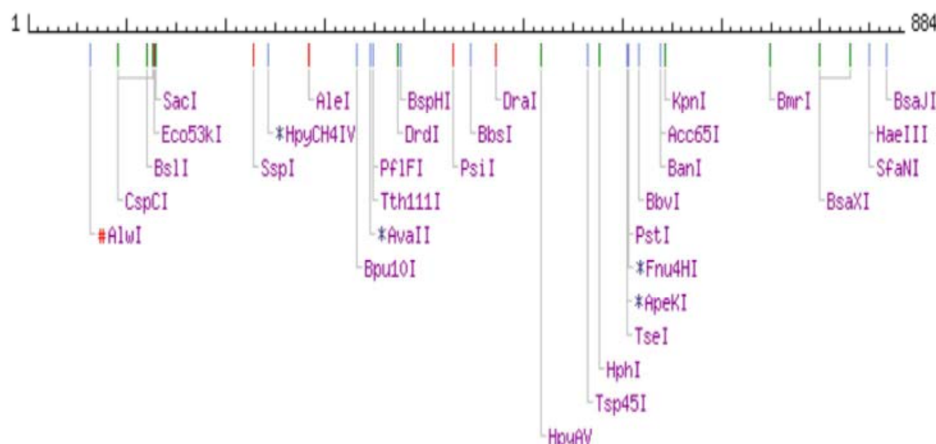

Fig. 12: Restriction enzymes present thought the H5 gene sequences.

Cloning of the Full Length H5 Gene: Molecular cloning of the amplified full length H5 gene has been performed in order to maintain the isolated full length gene for further studies. Selection and screening of treated competent *E. coli* standard strain using X-gal screening system provided at the used cloning kit (figure 9) indicated that the transformation process has been performed successfully. Moreover, in order to ensure the presence of the target PCR product, amplification of the full length H5 gene using the cloning vector isolated from the successful transformed cells as a template has been carried out *via* PCR as described above. As shown in figure 10, generated PCR product confirmed the presence of the target full length H5 gene compared to the negative control cells.

CONCLUSION

In the present study, the full length H5 gene of the studied Egyptian isolate has been successfully isolated and cloned at a molecular vector. Furthermore, the isolated full length gene has been sequenced and analyzed by bioinformatics software in order to characterize the target local H5N1 strain and to identify the restriction recognition sequences present at the partial generated H5 gene sequences. Moreover, an efficient real time PCR protocol specific to detect the Egyptian H5N1 strains has been developed. Generated results of both *in silico* and *in vitro* analysis indicated that developed diagnostic protocols has a good efficiency and accuracy.

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